

## ORIGINAL ARTICLE

# Immunoprofiling of oral squamous cell carcinomas reveals high p63 and survivin expression

IS Lauxen<sup>1</sup>, MG Oliveira<sup>1</sup>, PV Rados<sup>1</sup>, MW Lingen<sup>2</sup>, JE Nör<sup>3</sup>, M Sant'Ana Filho<sup>1</sup>

<sup>1</sup>Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; <sup>2</sup>Department of Pathology, Pritzker School of Medicine, University of Chicago, Chicago, IL; <sup>3</sup>Angiogenesis Research Laboratory, Department of Cariology, Restorative Sciences and Endodontics, School of Dentistry, University of Michigan, Ann Arbor, MI, USA

**BACKGROUND:** Cancer is a multifactorial disease composed of cells that show somatic mutations and epigenetic changes. The aim of this study was to investigate the expression of proteins involved in the development and maintenance of epithelia, cell cycle regulation, and apoptosis in human oral squamous cell carcinoma (OSCC) tissue samples.

**METHODS:** A tissue microarray containing 65 primary human OSCC specimens was immunolabeled for bcl-2, survivin, epidermal growth factor receptor (EGFR), p21, p53, p63, and cleaved caspase-3.

**RESULTS:** Samples were scored for percentage of positively stained tumor cells and staining intensity. A total immunostaining score was also calculated, using the product of percentage and intensity scores. All specimens showed high scores, > 75%, for p63 and survivin, and 75.4% of the specimens also presented high EGFR expression. All cases showed p53-positive cells. p21 showed a diffuse staining pattern. The percentage of cells positive for cleaved caspase-3 and bcl-2 was low.

**CONCLUSIONS:** The high frequency of tumor cells expressing p63 and survivin highlights the role of these proteins in the malignant transformation of oral epithelium. Collectively, our results suggest that p63 and survivin may constitute attractive targets for cancer therapy in patients with OSCC.

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**Keywords:** oral squamous cell carcinoma; apoptosis; survival; cell cycle; diagnostic markers

## Introduction

Cancer is recognized as a multifactorial disease composed of cells that show somatic mutations and epigenetic

changes (Grizzi *et al*, 2006). Oral cancer is a subgroup of head and neck cancers; it is the sixth most frequent type of cancer in the general population, with variable incidence rates across countries (Laimer *et al*, 2007). Despite advances in modern technologies, the 5-year relative survival rate of patients with oral cavity and pharynx cancer has shown only a modest improvement over the past few years (van der Waal, 2013).

At the individual level, the TNM classification does not reliably predict clinical outcomes or the histological grading of oral cancer in general and of oral squamous cell carcinomas (OSCC) in particular (Keski-Säntti *et al*, 2007). The relationship among key regulatory proteins of survival/death pathways and their differentiation may potentially have diagnostic and therapeutic value in OSCC.

The p63 protein is a transcription factor involved in epidermal development and maintenance (Candi *et al*, 2006). Its overexpression is associated with tumors known to harbor a high frequency of p53 mutations (Mirzayans *et al*, 2012). From an evolutionary perspective, the TP53 family comprises three members (TP53, TP63, TP73), probably deriving from the triplication of one ancestral gene (Hibi *et al*, 2000; Yang *et al*, 2002). The p53 transcription factor also plays a role in DNA damage. Similarly to protein p63, p53 can participate in both apoptosis and cell proliferation events (Yang *et al*, 1999; Hibi *et al*, 2000; Little and Jochemsen, 2002; Candi *et al*, 2008; van der Vorst *et al*, 2012).

The p21<sup>WAF1</sup> protein is a cyclin-dependent kinase inhibitor essential for the regulation of cell proliferation, differentiation, and apoptosis. The expression of p21 itself is regulated by p53, leading to either inhibition of cell cycle progression or apoptosis (Gomes *et al*, 2008; Mirzayans *et al*, 2012). p21 is also a substrate for caspase-3, a mediator of the intrinsic cell death pathway, able to activate mechanisms that prevent cell death via cleavage of classical caspase-3 (Warfel and El-Deiry, 2013). Caspases are modulated by several endogenous cellular factors, including inhibitor of apoptosis proteins (IAPs) (Burz *et al*, 2009). Survivin is an IAP and controls cell division by regulating mitotic activity at different levels. Upregulation

Correspondence: Isabel S. Lauxen, BSc, Department of Oral Pathology, School of Dentistry, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2492 Sala 503, Porto Alegre, RS, 90035-003, Brazil. Tel: +55 (51) 33085011, Fax: +55 (51) 33085439, E-mail: isabel.lauxen@ufrgs.br

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of survivin has been detected at the protein level in OSCCs (De Maria *et al*, 2009).

Bcl-2 acts as a key regulator of cell proliferation, differentiation, and survival. It is an anti-apoptotic protein that shows a high expression in several tumors (Németh *et al*, 2005).

Finally, epidermal growth factor receptor (EGFR) is involved in various cellular processes, including cell growth, motility, and invasion, angiogenesis, inhibition of apoptosis, and cell adhesion. EGFR may enhance the malignant potential of epithelial tissues via overexpression (Herbst, 2004; Katz *et al*, 2007; Laimer *et al*, 2007).

While most of these proteins have been studied individually in the context of oral cancer, their expression patterns have not been determined globally in human OSCCs. The aim of this study was the analysis of the expression of these proteins in a tissue microarray (TMA) containing 65 primary human OSCC specimens.

## Materials and methods

This study protocol was reviewed and approved by the Institutional Review Board of the institution where the study was carried out. Informed consent was obtained from all participants before sample collection.

### TMA preparation

The tissue microarrays were prepared using incisional biopsies of 65 patients with primary OSCC. None of the patients had undergone radiotherapy or chemotherapy prior to surgery. Data on age, gender, and lesion site are summarized in Table 1. Samples were formalin fixed and paraffin embedded. Hematoxylin and eosin-stained slides from each specimen were assessed by an experienced oral pathologist. Two tissue cylinders of 1-mm diameter, suitable for inclusion in the TMA, were punched from selected areas of each donor block and brought into a recipient block. Four samples had two donor blocks and one had three donor blocks. An automated tissue arrayer (ATA-27, Beecher Instruments Inc., Sun Prairie, WI, USA), from the Human Tissue Resource Center of the

University of Chicago, was used to make the recipient block. One recipient block was made, containing 142 OSCC cores and human samples for use as positive control cores (breast cancer, tonsil, lung cancer, and placenta tissues).

### Immunohistochemistry

Four- $\mu$ m thick sections were deparaffinized and rehydrated through ethanol. Antigen retrieval was performed, and sections were incubated with primary antibodies for bcl-2 (clone E17, Epitomics, 1:50), survivin (clone D8, Santa Cruz Biotechnology, 1:50), EGFR (clone 31G7, Zymed, 1:100), p21<sup>WAF1/Cip1</sup> (clone SX118, Dako, 1:25), p53 (clone DO-1, Calbiochem, 1:100), p63 [clone 4A4 + Y4A3(63PO2), Neomarkers 1:100], and cleaved caspase-3 (clone Asp 175, 5<sup>a</sup>1, Cell Signaling, 1:50), for 1 h at room temperature. The EnVision<sup>TM</sup>+ system (Dako, Carpinteria, CA, USA) and 3, 3'-diamino benzidine (Dako, Carpinteria, CA, USA) were used for visualization. For each core, images (400 $\times$  magnification) from all contiguous and consecutive fields were captured using a video camera (Q-Color5<sup>TM</sup>, Olympus America, Inc., Center Valley, PA, USA) coupled to a microscope. Images were recorded using the QCapture Pro<sup>TM</sup> 5 software (QImaging Co., Surrey, BC, Canada). A separate file was created for each immunolabel, and all images of the same core were stored in a subfile. Immunolabeled tumor cells were scored according to percentage of the total number of cells and graded semiquantitatively, as follows: 0% = 0, <25% = 1, 25–75% = 2, >75% = 3. Staining intensity was also evaluated semiquantitatively and was graded as follows: negative, grade 0; mild, grade +1; moderate, grade +2; and high staining intensity, grade +3. Finally, a total immunostaining score was calculated, using the product of percentage and intensity scores (the total score could range from 0 to 9). Tumors were randomly categorized into samples with high, low, and no expression, using the following cut-off values: 0 = no expression, 1–3 = low, and 4–9 = high expression. The immunolabeling area (nucleus, cytoplasm, or membrane, alone or combined) was observed and recorded. Slides were assessed by three oral pathologists (M.G.O., P.V.R., M.S.F.) previously calibrated, with acceptable intra-observer and interobserver kappa values (0.7–1.0).

### Statistical analysis

The Friedman non-parametric test, followed by the multiple comparison test, was used to analyze the total scores obtained for staining intensity vs. percentage of positive cells in the different immunolabels. Spearman's correlation coefficient was used to assess correlations between staining intensity scores and percentage of positive cells. Sixty-one samples had at least one score for each of the seven immunolabels and were considered suitable for analysis.

Intensity and positivity scores obtained for each immunolabel were analyzed in relation to patient gender using the Mann–Whitney non-parametric test, and in relation to patient age and lesion site using the Kruskal–Wallis non-parametric test. For all analyses,  $P < 0.05$  was considered statistically significant.

**Table 1** Patient characteristics

|                    | n  | %     |
|--------------------|----|-------|
| Gender             |    |       |
| Male               | 09 | 13.85 |
| Female             | 56 | 86.15 |
| Age (years)        |    |       |
| $\leq 50$          | 16 | 24.6  |
| 51–60              | 23 | 35.4  |
| 61–90              | 21 | 32.3  |
| NI                 | 05 | 7.7   |
| Lesion site        |    |       |
| Palate             | 05 | 7.7   |
| Lower lip          | 06 | 9.23  |
| Tongue             | 13 | 20    |
| Floor of the mouth | 20 | 30.77 |
| Gingiva            | 21 | 32.31 |

NI, not informed.

## Results

The analysis of patient characteristics revealed no significant differences in staining intensity scores and percentage of positive cells in relation to patient age and lesion site for all proteins investigated (data not shown). No gender differences were observed for staining intensity either. However, when analyzing percentage of positive cells, males showed significantly higher scores for p21 than females (male, mean rank: 31.35; female, mean rank: 17.94;  $P = 0.035$ ).

Figure 1 shows the total scores calculated for staining intensity vs. percentage of positive cells in each immunolabel. All specimens showed high p63 expression, >75%, and most of them displayed p63 positivity with gradual decreases from the basal layer toward the squamous layer. All specimens also showed high survivin expression, >75%, with most labeled cells located in the nucleus/cytoplasm. No relationship was observed between a large number of labeled cells and poorly differentiated carcinomas.

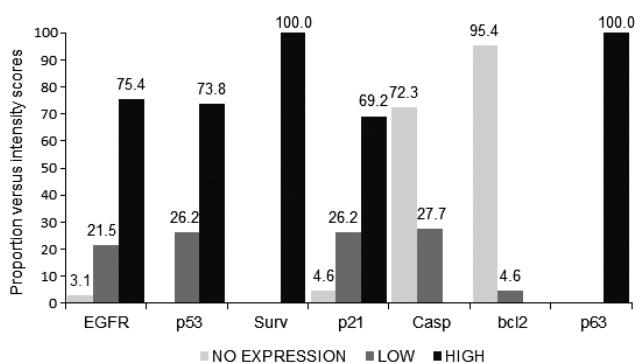
Forty-nine specimens (75.4%) showed high EGFR expression, predominantly located in the membrane and membrane/cytoplasm. Finally, all biopsies showed either low or high scores for p53, with 73.8% ( $n = 48$ ) of the cases showing high p53 labeling. The p53 protein revealed a predominantly nuclear and nuclear/cytoplasmic localization, and immunolabeled cells displayed a diffuse pattern. Higher numbers of labeled cells were observed in the most poorly differentiated carcinomas.

Neoplasms with keratin pearls were p63-, EGFR-, p53-, and survivin-positive only in their peripheral region. All samples analyzed for p21 expression showed a diffuse staining pattern. Cleaved caspase-3 and bcl-2 were the proteins with the lowest numbers of stained cells when compared with the other proteins investigated.

Spearman's correlation coefficient for percentage intervals, considering samples with at least one score for each immunolabel, revealed a negative correlation between p53 and cleaved caspase-3 and a positive correlation between p21 and p63 (Table 2).

## Discussion

In this sample, all specimens showed high levels of p63 and survivin expression, suggesting an involvement of



**Figure 1** Total scores for percentage vs. intensity of stained tumor cells. Cut-off values: 0 = no expression; 1–3 = low; 4–9 = high expression

these two proteins in the development and maintenance of oral cancer.

The p63 protein had already been shown to be involved in epithelial development by regulating stem cell/transient amplifying cells, their differentiation, and cell death (Yang *et al*, 1999; Little and Jochemsen, 2002; Candi *et al*, 2006). p63 encodes at least six isoforms, divided in two groups: TAp63, involved in apoptosis, and  $\Delta$ Np63, involved in cell proliferation (Yang *et al*, 1999, 2002; Little and Jochemsen, 2002; Gurgel *et al*, 2008). Our results corroborate previous findings regarding the role played by this protein in the regulation of epithelial differentiation (Gurgel *et al*, 2008; Rosenbluth *et al*, 2009): in our sample of OSCC specimens, p63 was found in all epithelial layers, with gradual decreases from the basal toward the squamous layer.

Survivin has also been detected in the cytoplasm and nucleus of cancer cells (Qi *et al*, 2010), in line with our findings. Cytoplasmic survivin has been characterized as anti-apoptotic and associated with microtubules, directly or indirectly interfering with the function of caspases; nuclear survivin, in turn, is suspected to control cell division (Qi *et al*, 2010).

The proteins assessed in the present study, namely p63, p53, p21, survivin, bcl-2, and EGFR, are directly involved in the inhibition or induction of apoptosis. Immunolabeling of cleaved caspase-3 suggests that an apoptotic event has occurred. Based on our findings and on relevant literature, we hypothesize that, in our sample, p21 and survivin downregulated apoptosis, influenced by p63 and p53.

Abnormal accumulation of p53 is a common finding in the development of oral carcinomas (Bidaud *et al*, 2010). Mutations in the TP53 gene are the abnormalities most frequently found in OSCCs. Notwithstanding, most of these mutations result in gains of function, including the ability to interact with mitochondrial caspase-3, establishing an apoptosis-resistant phenotype (Mirzayans *et al*, 2012). We believe that this mechanism could explain, at least in part, the negative correlation observed between p53 and cleaved caspase-3.

To block the cell cycle, p53 induces transcription of the p21<sup>WAF1</sup> protein. Furthermore, when p21<sup>WAF1</sup> binds to PCNA, it directly inhibits DNA replication during the S phase (Whyte *et al*, 2002). Finally, p21<sup>WAF1</sup> immunolabeling is altered during oral carcinogenesis, increasing as the severity of histological findings progresses (Weinberg and Denning, 2002; Choi *et al*, 2003; Warfel and El-Deiry, 2013).

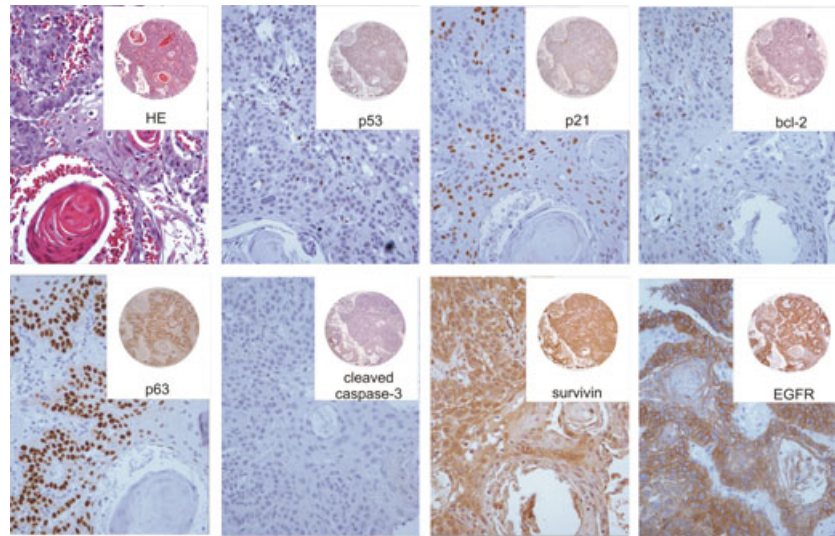
Our results show a significant direct correlation between p63 and p21, which may be explained by a p53-independent induction of p21<sup>WAF1</sup> in response to cell differentiation signals – it should be noted that p21 also has a role in maintaining stem cell quiescence, acting as an oncogene (Patel *et al*, 2009; Warfel and El-Deiry, 2013).

Another gene that may be induced by gain-of-function mutations in p53 proteins is EGFR. In our study, two specimens (3.1%) failed to show EGFR expression, and 75.4% of the samples showed highly stained tumor cells, a result similar to that reported by Laimer *et al* (2007), Monteiro *et al* (2010).

**Table 2** Spearman's correlation coefficient for percentage intervals considering the 61 specimens with at least one score for each immunolabel

|                     | <i>Epidermal growth factor receptor (EGFR)</i> | <i>p53</i> | <i>Survivin</i> | <i>p21<sup>WAF1</sup></i> | <i>Cleaved caspase-3</i> | <i>Bcl-2</i> |
|---------------------|--|------------|-----------------|---------------------------|--------------------------|--------------|
| p53                 | -0.019   | -          | -               | -                         | -                        | -            |
| Survivin            | -0.139   | -0.023     | -               | -                         | -                        | -            |
| p21 <sup>WAF1</sup> | 0.093  | 0.107      | 0.028           | -                         | -                        | -            |
| Cleaved caspase-3   | -0.177   | -0.252*    | 0.101           | -0.026                    | -                        | -            |
| bcl-2               | 0.113  | 0.126      | 0.042           | -0.152                    | -0.082                   | -            |
| p63                 | 0.229  | -0.023     | -0.052          | 0.284*                    | 0.101                    | 0.042        |

\*Significant correlation at 5%.



**Figure 2** Hematoxylin and eosin-stained oral squamous cell carcinoma (OSCC) specimens: p53, p21<sup>WAF1</sup>, bcl-2, p63, cleaved caspase-3, survivin, and epidermal growth factor receptor (EGFR) immunolabeling

Functional dissection of the bcl-2 family reveals proteins with primary anti-apoptotic (bcl-2, bcl-x1, bcl-w, and Mcl-1, among others) vs. pro-apoptotic (Bax, Bak, Bad, Bok, and Bid, among others) roles (Mallick *et al*, 2009; Patel *et al*, 2009). The anti-bcl-2 antibody used in our study does not cross-react with other bcl-2 protein family members. Differently from various malignant lesions that show a high percentage of bcl-2-positive cells (Patel *et al*, 2009), 95.4% of our specimens did not show bcl-2 immunopositivity. We speculate that many of the studies showing high bcl-2 immunolabeling in OSCCs may have used antibodies that cross-react with other members of this family. Our results revealed a higher percentage of cleaved caspase-3 than of bcl-2 immunolabeling, and none of the three bcl-2-positive samples was positive for cleaved caspase-3. These results lead to the hypothesis that, in this sample, the anti-apoptotic bcl-2 protein may not have participated in the pathway that suppresses apoptosis in tumor cells – again, it should be noted that another property of mutated p53 is to downregulate the transcription of some genes, including those that encode bcl-2 (Mirzayans *et al*, 2012).

Previous studies (Putti *et al*, 2002; Choi *et al*, 2003; Tanaka *et al*, 2003; Bascones *et al*, 2005; Kummoona *et al*, 2007; Bidaud *et al*, 2010) were the basis for the immunohistochemistry pattern of the proteins investigated in normal mucosa epithelium. Non-neoplastic tissues adjacent to tumors were not included in this study because they could

present abnormalities regardless of their morphology. Our belief is based on the field cancerization theory proposed by Slaughter *et al* (1953) and widely studied until today.

As shown in the present study, immunohistochemistry provides valuable information that can guide treatment decisions in the management of cancer patients (Figure 2). Collectively, our findings highlight the role played by p63 and survivin in the malignant transformation of oral epithelium and indicate that they could be attractive targets for cancer therapy in patients with OSCC.

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#### Conflict of interest

None declared.

#### Author contributions

Isabel Lauxen: designed and performed experiments, constructed the TMA, developed the immunohistochemistry, analysed data and wrote the manuscript, edited the manuscript; Mark Lingen: technical support and conceptual advice, analysed data, revised the manuscript; Jacques Nor: analysed data, revised the manuscript; Manoel SantAna Filho: supervised the project, read the immunohistochemistry slides, analysed data, revised the manuscript;

Pantelis V. Rados-read the immunohistochemistry slides, analysed data, revised the manuscript; Marcia G. Oliveira: selected the cores to the TMA, read de immunohistochemistry slides, analysed data, wrote the manuscript.

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